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Formation of *o*-Tyrosine and Dityrosine in Proteins during Radiolytic and Metal-catalyzed Oxidation*

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To evaluate their usefulness as chemical indicators of cumulative oxidative damage to proteins, we studied the kinetics and extent of formation of *ortho*-tyrosine (*o*-Tyr), dityrosine (DT), and dityrosine-like fluorescence (E_x = 317 nm, E_m = 407 nm) in the model proteins RNase and lysozyme exposed to radiolytic and metal-catalyzed (H_2O_2/Cu^{2+}) oxidation (MCO). Although there were protein-dependent differences, *o*-Tyr, DT, and fluorescence increased coordinately during oxidation of the proteins in both oxidation systems. The contribution of DT to total dityrosine-like fluorescence in oxidized proteins varied from 2–100%, depending on the protein, type of oxidation, and extent of oxidative damage. In proteins exposed to MCO, DT typically accounted for >50% of the fluorescence at DT wavelengths. These studies indicate that *o*-Tyr and DT should be useful chemical markers of cumulative exposure of proteins to MCO *in vitro* and *in vivo*.

Oxygen radicals are formed ubiquitously in biological systems by both enzymatic and metal-catalyzed oxidation (MCO)[1] reactions (1). These radicals may react with and modify neighboring molecules, and there is evidence that oxidative modification of proteins may be physiologically important, serving as a "marking" step for initiating protein degradation (2–4). There is increasing evidence that oxidative stress, resulting from either excessive oxygen radical production or compromised anti-oxidant defenses, may be involved in the natural aging of tissue proteins as well as the pathogenesis of atherosclerosis, ischemic-reperfusion injury, inflammatory diseases, and diabetes (5–8). Oxidation of proteins by microsomal oxidases and MCO treatment is known to produce carbonyl modifications of amino acids, and protein-bound carbonyl groups are observed to increase in tissue proteins with age and in cultured cells with donor age and population doubling number (2–4). Increased levels of protein carbonyl groups have also been reported in fibroblasts cultured from progeric patients and following ischemic-reperfusion injury (2). However, only trace levels of carbonyl compounds are detectable in tissue proteins, undoubtedly because of the reactivity of aldehydes and ketones with nucleophiles under physiological conditions. Increases in levels of these compounds with age and pathological conditions are also limited, generally less than 2-fold, consistent with their reactivity and possible role as markers for the catabolism of proteins. For these reasons, carbonyl compounds may be useful as indicators of steady-state oxidative stress to protein, but may not be good candidates for assessing cumulative damage to long-lived proteins by oxidation reactions.

In contrast to the lability of carbonyl compounds, some products of oxidation of Phe and Tyr are stable, even to acid hydrolysis of proteins. These include dityrosine (DT) and *ortho*-tyrosine (*o*-Tyr) which are formed during radiolytic oxidation of proteins (9–12) and, during limited radiolysis, increase gradually in protein as a function of absorbed dose (Reaction 1). While DT may also be formed in proteins by enzymatic oxidation (13), there is no information on formation of *o*-Tyr under these conditions, and, in general, there is little information on the formation of *o*-Tyr and DT in proteins exposed to MCO treatment or comparative formation of these oxidation products during radiolytic and MCO. In the present work, we set out to develop sensitive and specific assays for these amino acid oxidation products and to evaluate their usefulness as markers of cumulative oxidative modification of protein. RNase and lysozyme were chosen as model proteins for the studies since they are chemically and structurally well characterized. Additionally, they provide a valuable contrast since RNase lacks Trp while lysozyme is relatively rich in this amino acid (6 mol of Trp/mol); thus, stable products of oxidation of Trp might also be identifiable for further study. Since DT is a fluorescent compound and oxidation of protein is accompanied by an increase in DT-like, alkaline blue-green fluorescence (E_x = 317 nm, E_m = 407), we also addressed the contribution of DT to the total DT-like fluorescence in oxidized proteins. The results presented below indicate that *o*-Tyr and DT are sensitive indicators of a protein's cumulative exposure to both radiolytic oxidation and MCO and suggest that these compounds should be useful as indicators of progressive oxidative damage to proteins *in vitro* and *in vivo*. In the accompanying paper (14), we describe results of measurement of these compounds, as well as DT-like fluorescence, in human lens protein as a function of age.

EXPERIMENTAL PROCEDURES

Materials—Unless otherwise indicated, reagents were of the highest quality obtainable from either Sigma or Aldrich Chemical Co. RNase (type XII-A), lysozyme (grade 1), horseradish peroxidase (type II), bovine liver catalase (catalog C-30), and chymotrypsin (type I-S) were purchased from Sigma, and d_5-L-phenylalanine (98% pure) from Cambridge Isotope Laboratories (Woburn, MA).

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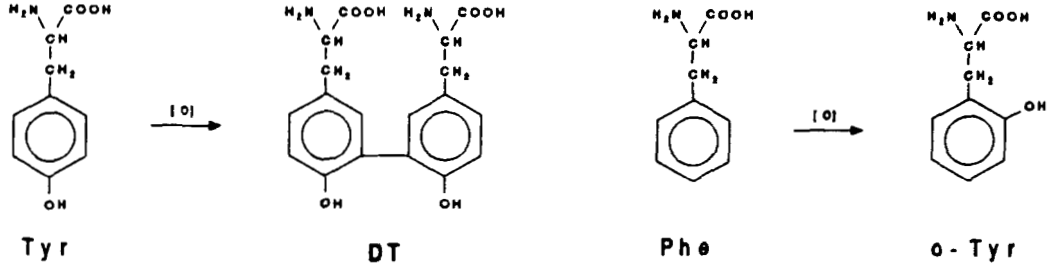
[1] The abbreviations used are: MCO, metal-catalyzed oxidation; *o*-Tyr, *ortho*-tyrosine; DT, dityrosine; HFBA, heptafluorobutyric acid; RP-HPLC, reversed-phase high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; SIM-GC/MS selected ion monitoring-gas chromatography/mass spectrometry.



REACTION 1

Preparation of Standards—*o*-Tyr, deuterated at four positions on the phenyl ring (d_4-*o*-Tyr), was prepared by oxidation of d_5-L-Phe with H_2O_2 in the presence of Cu^{2+}. Briefly, 25 mg of deuterated Phe was dissolved in 25 ml of deionized water, and the solution was made 100 mM in H_2O_2 and 1 mM in $CuSO_4$. The mixture was adjusted to pH 9.0 with 0.1 N NaOH and incubated overnight at 37 °C. The reaction was quenched by adjusting to pH 6.0 with 1 N HCl. Selected ion monitoring-gas chromatography/mass spectrometry (SIM-GC/MS) was used to detect d_4-*o*-Tyr (see below), and its concentration was determined by amino acid analysis using authentic *o*-Tyr as standard. The d_4-*o*-Tyr was used as internal standard for GC/MS assays without further purification.

DT was synthesized as described by Ushijima *et al.* (15) by oxidation of L-Tyr with horseradish peroxidase and H_2O_2 and was purified by reversed-phase HPLC (RP-HPLC) using conditions described below for analysis of the DT content of oxidized proteins. The concentration of DT in standard solutions was determined by the trinitrobenzenesulfonic acid assay, as described by Spadaro *et al.* (16), using Tyr as standard (2 mol of Tyr/mol of DT).

Oxidation of Proteins—RNase A and lysozyme were oxidized by both radiolytic and MCO reactions. For radiolytic reactions, protein (10 mg) was dissolved in deionized water (0.5 mg/ml) and exposed to oxygen radicals produced by 18 MV x-rays generated by a linear accelerator (Varian Clinac 20, Varian Instruments). The absorbed dose rate was 5 ± 0.05 kilorads/min as measured by Fricke-Hart dosimetry (17); exposure time was varied from 0 to 15 min, yielding total doses of 0–75 kilorads. The radiation doses (15–75 kilorads) were based on those used by Davies and colleagues (18–20) in studies on effects of radiolytic oxidation (0.5–100 kilorads) on susceptibility of proteins to proteolytic degradation. For MCO, protein (10 mg) was dissolved in deionized water (0.5 mg/ml) and incubated at room temperature with H_2O_2 (0–10 mM) in the presence of 0.1 mM $CuSO_4$ as catalyst. After 4 h, the MCO reactions were quenched by the addition of 1 mM diethylenetriaminepentaacetic acid and 170 units of catalase. Control MCO samples were treated identically with oxidized samples, except that H_2O_2 was omitted from the incubations. The concentrations of H_2O_2 used in the MCO experiments (0.5–10 mM) were also in the range typically used by other investigators (4, 21). Data shown in the figures and tables are typical of 3–4 independent experiments.

Protein Hydrolysis—Following oxidation reactions, control and treated samples were dried *in vacuo* using a Savant Speed-Vac concentrator (Savant Instruments, Farmingdale, NY) and then resuspended at 5 mg of protein/ml of deionized water. Proteins (2 mg) were hydrolyzed *in vacuo* in 2 ml of 6 N HCl for 24 h at 110 °C. The hydrolysates were dried *in vacuo* to remove acid, then resuspended in 200 μl of deionized water, and separate aliquots were taken for amino acid analysis and determination of *o*-Tyr and DT. When known amounts of authentic *o*-Tyr or DT were mixed with native proteins, 90–95% of added amino acid was recovered (see below), indicating that the conditions used for protein hydrolysis did not generate or cause significant losses of these oxidation products. Similarly, addition of Phe and Tyr in amounts equivalent to that in protein did not increase the amounts of *o*-Tyr or DT, respectively, in the hydrolysates of native proteins.

Measurement of o-Tyr in Oxidized Proteins—The *o*-Tyr content of hydrolyzed proteins was determined following addition of the deuterated internal standard and conversion of amino acids to their *N,O*-acetyl isopropyl ester derivatives. Briefly, 250 ng of d_4-*o*-Tyr was added to the hydrolysate (~1 mg of protein), and the sample was dried *in vacuo*. For preparation of the isopropyl esters, the sample was dissolved in 1 ml of 1 N isopropanolic HCl and heated for 30 min at 110 °C. Solvent was evaporated under a stream of nitrogen, and the product was redissolved in 0.8 ml of pyridine. Acetic anhydride (0.2 ml) was then added, and the mixture was incubated at room temperature for 30 min to obtain the *N,O*-acetyl derivatives. After removal of the reagents under a stream of nitrogen, the sample was redissolved in 100 μl of ethyl acetate.

A 2-μl aliquot of the derivatized hydrolysate was injected for analysis by SIM-GC/MS using a Hewlett-Packard Model 5890 gas chromatograph equipped with a Model 7673A Autosampler and Model 5970 Mass Selective Detector (Hewlett-Packard). A 30-m DB-5 capillary column (J & W Scientific, Folsom, CA) was used with the following temperature program: 2 min at 100 °C, ramp to 260 °C at 5 °C/min, ramp to 290 °C at 15 °C/min, hold for 3 min at 290 °C. The full scan mass spectrum of derivatized *o*-Tyr contained the following major ions: m/z 307 = M^+; m/z 265 = $M^+ - H_2C{=}C{=}O$; m/z 206 = $M^+ - (H_2C{=}C{=}O + C_3H_7O)$; m/z 146 = $M^+ - (H_2C{=}C{=}O + C_3H_7O + CH_3COOH)$ (data not shown). Although the molecular ion was visible in the spectrum, the more intense m/z = 265 ion was chosen for quantitation and normalized to the m/z = 269 ion of the internal standard, d_4-*o*-Tyr; similar results were obtained using m/z = 146 and 150 ions, which were monitored simultaneously. Quantitation was based on external calibration using a standard curve prepared with increasing amounts of *o*-Tyr in the presence of a constant amount of d_4-*o*-Tyr.

To correct for variations in protein content, the *o*-Tyr measured in each sample was normalized to the Val content of an aliquot of the original hydrolysate, determined by amino acid analysis; the Val content of the proteins was unchanged during the oxidation reactions. Amino acid analyses were performed on a Waters HPLC amino acid analyzer system using a cation exchange column, as described previously (22). Final *o*-Tyr concentrations are expressed per mol of Phe in the orginal protein, based on the molar ratio of Val:Phe in the protein. In this manner, the measurements were not affected by the decrease in Phe in the protein during the oxidation reactions.

Measurement of DT in Oxidized Proteins—An aliquot (1–10 μl) of the protein hydrolysate was diluted to 200 μl with 2% heptafluorobutyric acid (HFBA) and analyzed by RP-HPLC. RNase was analyzed using a Varian 5500 chromatograph (Varian Instruments, Sunnyvale, CA) and lysozyme with a Varian 5000 chromatograph, each fitted with a Supelco (Supelco, Bellefonte, PA) C-18 column (Supelcosil LC-318; 250 × 4.6 mm, 5 μm). Buffer A was 0.1% aqueous HFBA and buffer B, 0.1% HFBA in 50% acetonitrile. The gradient program used for DT analysis in RNase was: zero time, 100% A; 20 min, 10% B; 48 min, 31% B; 53 min, 100% B. The gradient program used for DT analysis in lysozyme was: zero time, 100% A; 20 min, 15% B; 50 min, 22% B; 53 min, 100% B. A different gradient was required for the analysis of DT in lysozyme because of the greater complexity of the RP-HPLC profile obtained following radiolysis of this protein (Fig. 2, below).

The DT content of proteins was determined by measuring DT fluorescence at alkaline pH after mixing the column effluent (1 ml/min flow rate) with an equal volume of 25 mM potassium phosphate, pH 11.5. The final pH was 11.2, and the eluate was monitored for fluorescence at E_x = 317 nm, E_m = 407 nm using a Shimadzu RF5000U spectrofluorophotometer (Shimadzu Corp., Tokyo, Japan) for RNase and a Gilford Fluoro IV Spectrofluorometer (Gilford, Oberlin, OH) for lysozyme. Peak areas were integrated using a Hewlett-Packard 3390A recording integrator, and the assay was standardized by external standardization using a calibration curve generated with synthetic DT. To correct for variations in protein content, DT concentration was normalized to the Val content of each sample measured by amino acid analysis as above. Final DT concentrations were expressed per mol of Tyr in the orginal protein using the molar ratio of Val:Tyr in the protein.

Measurement of Protein Fluorescence—For measurement of total protein fluorescence at DT wavelengths, protein (1 mg/ml) was digested with 50 μg of chymotrypsin for 24 h at 37 °C in 50 mM phosphate buffer, pH 8.5. An aliquot of the digested protein, typically

5–25 μg, was then injected directly onto the HPLC system using a column-bypass valve for measurement of total fluorescence at E_x = 317 nm, E_m = 407 nm. Fluorescence readings were normalized to the Val content of each sample, measured by amino acid analysis. For construction of three-dimensional fluorescence plots, fluorescence spectra were acquired using a Shimadzu RF5000U spectrofluorophotometer interfaced to a laboratory computer with Lotus Measure (Lotus Development Corp., Cambridge, MA). Data were stored in Lotus 1-2-3 spreadsheet files and imported into Plot-It (Scientific Programming Enterprises, Haslett, MI) to generate three-dimensional fluorescence plots.

Gel Electrophoresis—Polyacrylamide gel electrophoresis (14% cross-linking) was performed in the presence of sodium dodecyl sulfate (SDS-PAGE) under reducing conditions as described by Laemmli (23). Proteins were detected by staining with Coomassie Brilliant Blue R-250.

RESULTS

Effects of Oxidation on Protein Structure and Amino Acid Composition—The effects of radiolytic and MCO treatment on the integrity of RNase and lysozyme were evaluated by SDS-PAGE. Oxidation of both proteins was accompanied by a dose-dependent decrease in the intensity of the monomer band, and an increase in staining in the low molecular weight region of the gels, but discrete products were not detected (data now shown). Amino acid analyses of the oxidized proteins indicated that, among the amino acids measured (*i.e.* excluding Pro, Trp, and Cys), only His, Phe, and Tyr were partially destroyed (Table I).

Formation of o-Tyr during Oxidation of Proteins—*o*-Tyr was not detectable in the native proteins, but accumulated in radiolyzed and MCO-treated RNase and lysozyme in a dose-dependent manner (Fig. 1). The yield of *o*-Tyr varied with the protein and method of oxidation, with lysozyme yielding greater fractional amounts of *o*-Tyr (per mol of Phe) in both oxidation systems; both proteins contain 3 mol of Phe/mol of protein. Under the conditions studied, *o*-Tyr accounted for up to 5% of the original Phe content of the proteins and 10–50% of the Phe destroyed during the oxidation reactions (Table I).

Formation of DT during Oxidation of Proteins—DT was also not detectable in native RNase or lysozyme, but was readily identified by RP-HPLC in hydrolysates of the oxidized proteins (Fig. 2). Identification of the DT peak was confirmed by its co-elution with authentic DT in mixing experiments (not shown), its fluorescence spectrum and decrease in fluorescence at acid pH, and its mass spectrum (the latter using methods described in the accompanying paper (14)). The chromatograms in Fig. 2, *A* and *C*, indicate that in addition to DT a number of DT-like fluorescent products, presently uncharacterized, are formed during radiolytic oxidation of the proteins. DT was, in fact, only a minor contributor to the total alkaline blue-green fluorescence generated during radiolytic oxidation, and the number of products and their relative yield from the two proteins were quite different. In contrast, on MCO treatment of the proteins (Fig. 2, *B* and *D*), DT accounted for the majority of fluorescence in both proteins and nearly quantitatively for the DT-like fluorescence in RNase, which lacks Trp. The yield of DT was dependent on dose for both proteins in both oxidation systems, but was 20–50-fold greater in the MCO system (Fig. 3). For MCO-treated proteins, DT accounted for up to 2% of the total Tyr content of the protein (Fig. 3) and up to 10% of the Tyr destruction (Table I). Thus, DT, like *o*-Tyr, appears to be a sensitive indicator of exposure of these proteins to MCO treatment.

Increase in Alkaline Blue-Green Fluorescence during Oxidation of Proteins—Fig. 4 compares the dose and concentration dependence of generation of fluorescence at DT wavelengths in RNase and lysozyme during exposure to radiolysis and MCO treatment. Lysozyme yielded higher levels of fluorescence than RNase in both oxidation systems, while MCO treatment yielded higher absolute levels of fluorescence for both proteins. The higher total fluorescence in lysozyme compared to RNase may result from the presence of Trp and its oxidation products in lysozyme. Fig. 5 summarizes the fractional contribution of DT to the total DT-like fluores-

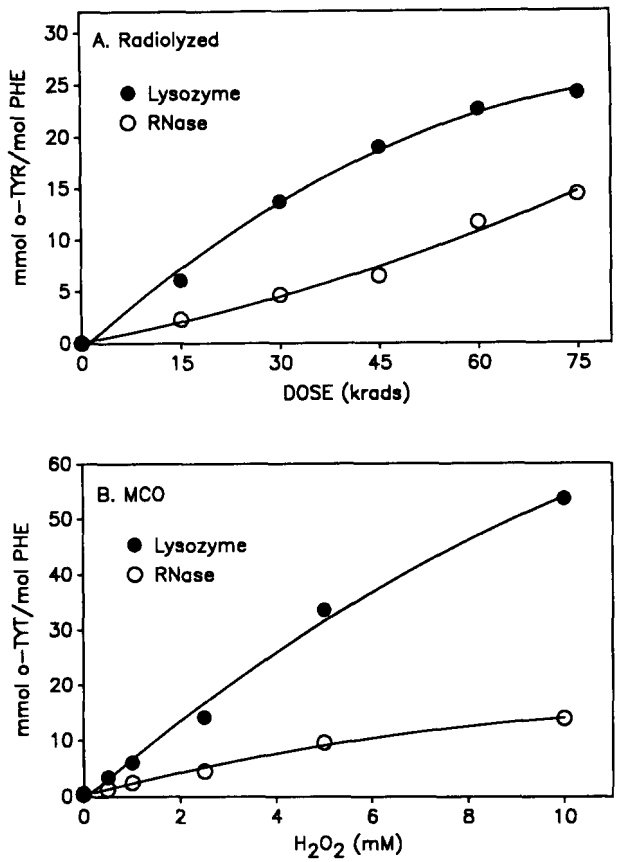


FIG. 1. **Dose-dependent accumulation of *o*-Tyr in irradiated (*A*) and MCO-treated (*B*) RNase (○) and lysozyme (●).** Proteins were oxidized and *o*-Tyr was measured by SIM-GC/MS as described under "Experimental Procedures." Data are typical of results obtained in 3–4 separate experiments.

TABLE I

Recovery of selected amino acids following radiolytic and metal-catalyzed oxidation of RNase and lysozyme

Amino acid analysis was carried out on oxidized proteins as described under "Experimental Procedures." The number of Phe, Tyr, and His residues in RNase is 3, 6, and 4, and in lysozyme is 3, 3, and 1, respectively.

Radiolysis	RNase			Lysozyme		
	Phe	Tyr	His	Phe	Tyr	His
krad	*% remaining*			*% remaining*		
0	100.0	100.0	100.0	100.0	100.0	99.8
15	99.3	83.1	96.8	99.1	97.6	96.9
30	100.6	80.6	96.9	98.8	90.5	92.7
45	99.6	73.6	96.2	96.2	89.7	88.6
60	96.2	64.5	96.4	92.5	84.3	83.5
75	94.6	68.0	97.0	91.3	83.9	81.1
MCO						
μM H_2O_2						
0	100.0	100.0	99.9	100.0	100.0	100.2
0.5	96.7	99.4	98.0	102.9	95.9	80.6
1.0	99.1	101.7	95.8	99.3	90.8	70.0
2.5	98.8	100.6	90.2	99.7	69.8	68.2
5.0	93.5	95.7	81.4	96.8	85.2	53.6
10.0	89.0	82.8	70.5	89.8	61.4	44.2

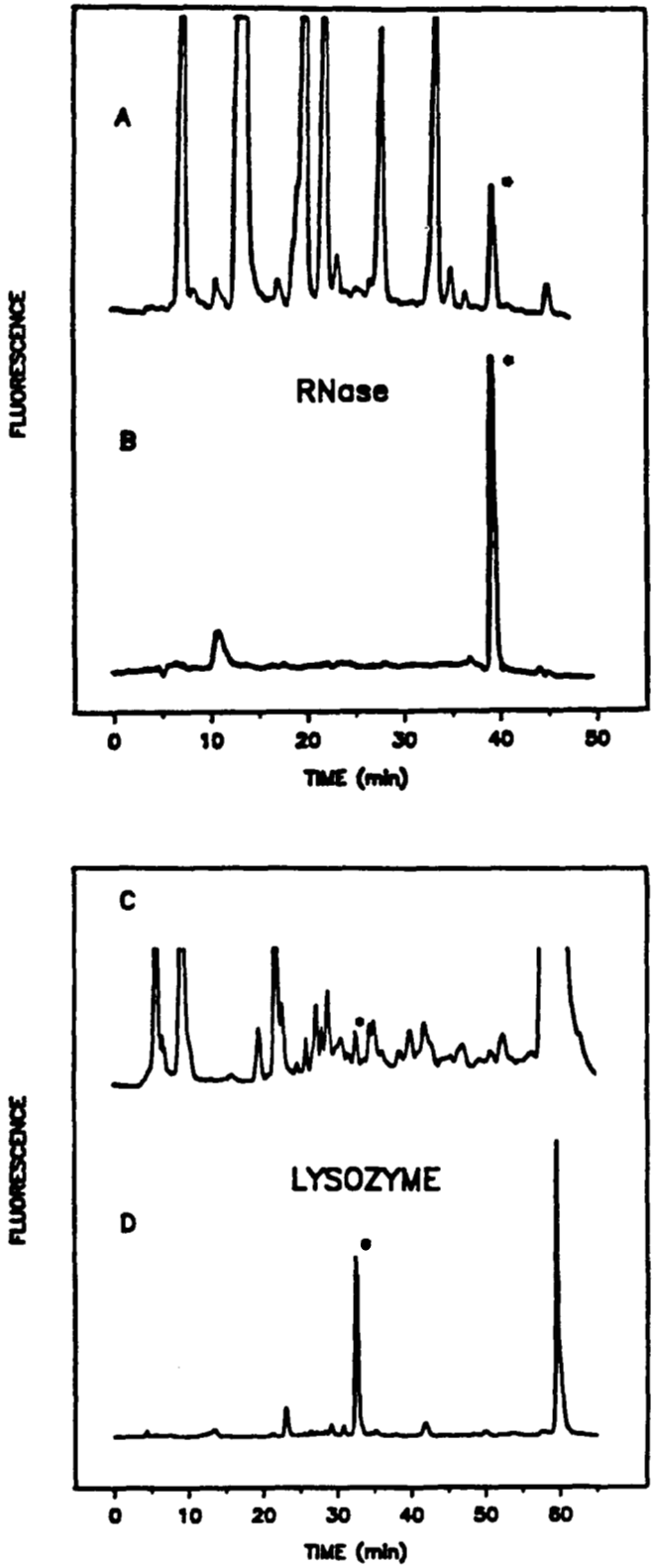


FIG. 2. **Identification of DT in acid hydrolysates of irradiated and MCO-treated RNase (*upper panel*) and lysozyme (*lower panel*).** DT was measured in protein hydrolysates by RP-HPLC using post-column fluorescence detection (E_x = 317 nm and E_m = 407 nm) as described under "Experimental Procedures." *Panels A* and *C* are chromatograms obtained for RNase and lysozyme, respectively, irradiated with 45 kilorads; *panels B* and *D* are chromatograms for RNase and lysozyme, respectively, after MCO with 2.5 mM H_2O_2. Scale for irradiated lysozyme in *panel C* is expanded 10-fold compared to the scale for the MCO-treated protein in *panel D*. *Asterisk* (*) indicates elution position of authentic DT in the different chromatographic systems used for analysis of each protein.

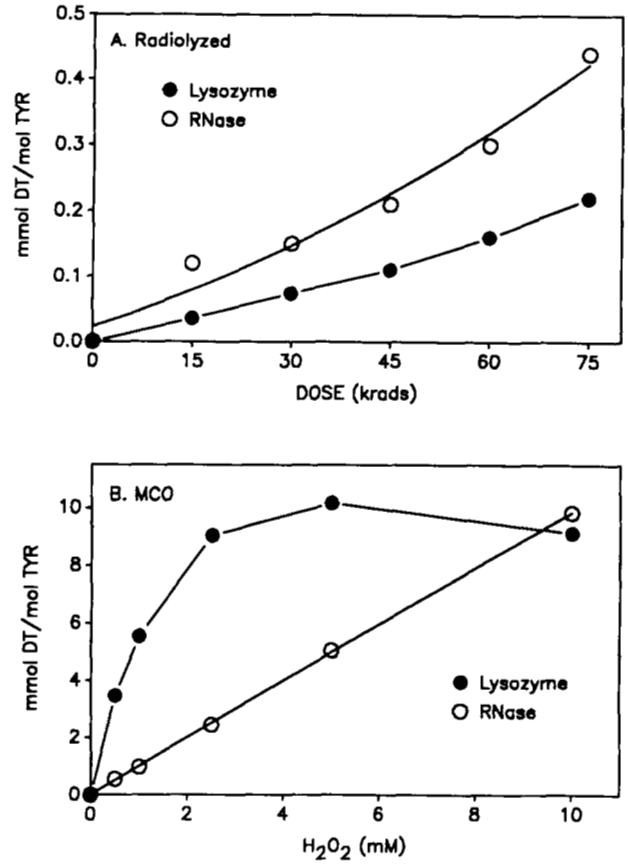


FIG. 3. **Dose-dependent accumulation of DT in irradiated (*A*) and MCO-treated (*B*) RNase (○) and lysozyme (●).** Proteins were oxidized, and DT was measured by RP-HPLC as described under "Experimental Procedures." Data are typical of results obtained in 3–4 separate experiments.

cence accumulating in the proteins during oxidation. Since acid hydrolysis yielded a 4–10-fold increase in total fluorescence in the hydrolysate compared to the oxidized protein, measurements of total fluorescence were carried out on chymotrypsin-digested proteins. This treatment was performed in order to eliminate effects of protein structure on fluorescence, although the total fluorescence of the protein was not significantly affected (<10% change) by the proteolytic treatment. The results in Fig. 5 support the general observation in Fig. 2 that DT accounted for only a fraction of the total DT-like fluoresence formed during radiolytic oxidation of the proteins, whereas it was the major fluorophore in protein oxidized by MCO treatment, accounting for essentially 100% of the fluorescence in MCO-treated RNase.

Spectrum of Alkaline Fluorescence Generated during Oxidation of Proteins—The three-dimensional fluorescence plots shown in Fig. 6 illustrate that although there is some variability in the visible wavelength fluorescence maxima of oxidized proteins, in general, the maxima for oxidized proteins are similar to those of DT (E_x = 317, E_m = 407). Trp fluorescence is particularly apparent in native lyzozyme (Fig. 6*B*), and its near absence from the spectrum of the oxidized protein (Fig. 6, *D* and *F*) is consistent with rapid destruction of Trp during oxidation. Although the fluorescent maxima of MCO and radiolyzed RNase and lysozyme are similar, the shapes of the spectra of irradiated protein are more complex than their MCO counterparts. The similarity of the fluorescent maxima of oxidized RNase and lysozyme indicate that fluorescent products originating from the oxidation of Trp did not significantly alter the overall fluorescent maxima of lysozyme compared to RNase.

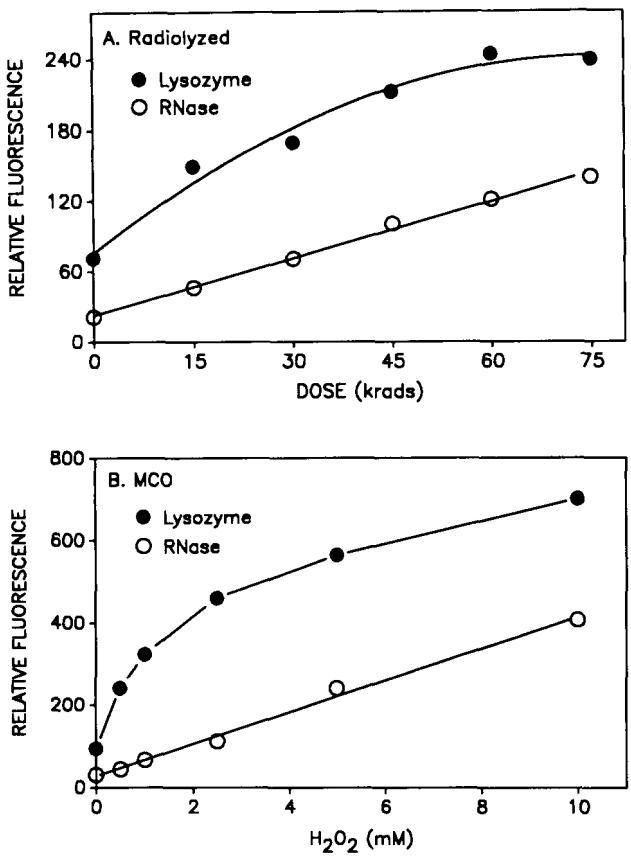


FIG. 4. **Dose-dependent formation of DT-like fluorescence in irradiated (*A*) and MCO-treated (*B*) RNase (○) and lysozyme (●).** Proteins were digested with chymotrypsin, and total fluorescence was measured at E_x = 317 nm and E_m = 407 nm as described under "Experimental Procedures." Data are typical of results obtained in 2–3 separate experiments.

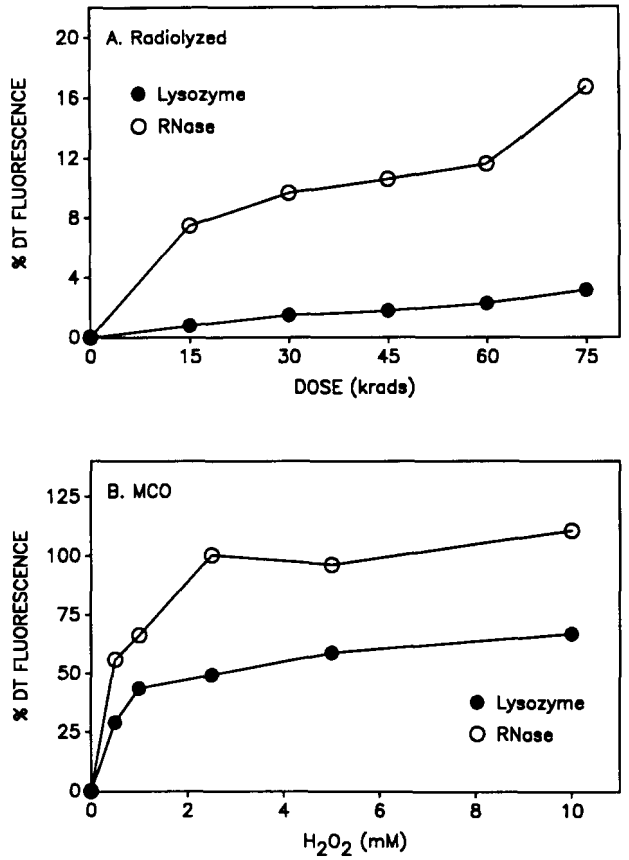


FIG. 5. **Contribution of DT to the total DT-like fluorescence in irradiated (*A*) or MCO-treated (*B*) RNase (○) and lysozyme (●).** Actual fluorescence of DT, measured by RP-HPLC, is expressed as a fraction of total fluorescence in the protein at DT wavelengths.

DISCUSSION

The long term goal of this research is to identify useful biomarkers of oxidative damage to proteins *in vivo*. To address this question we have developed assays for two chemical indicators of radiolytic and metal-catalyzed oxidation of protein, *o*-Tyr, and DT, which are produced by the oxidative modification of Phe and Tyr, respectively. These studies show that *o*-Tyr, DT, and DT-like fluorescence increase gradually in proteins during exposure to both radiolytic and MCO treatment, and that DT is a major "alkaline blue" fluorophore in proteins oxidized by MCO treatment, even in lysozyme which is rich in Trp residues. Comparison of the SDS-PAGE analyses (data not shown) and the *o*-Tyr and DT analyses (Figs. 1 and 3) indicates that both *o*-Tyr and DT are detectable in protein prior to extensive cleavage and fragmentation of the protein. Overall, these findings indicate that both *o*-Tyr and DT should be useful as markers of oxidative damage to protein since neither is present in the native protein, and both accumulate in protein in a dose-dependent manner during exposure to oxidative stress.

Although the amounts of *o*-Tyr formed in RNase and lysozyme were comparable during radiolytic and MCO treatment, the yield of DT was 20–50 times higher in MCO-treated RNase and lysozyme than in the radiolyzed proteins. The difference in DT yield does not appear to result from greater oxidative damage to protein by MCO treatment, since SDS-PAGE (data not shown) and amino acid analysis (Table I, loss of Tyr) of RNase and lysozyme show that similar extents of destruction occurred during the two treatments. Stadtman (2) and Hunt and Wolff (24) have noted that damage mediated by MCO treatment of protein is a site-specific process, occurring in regions where transition metals are complexed to the protein. The increased DT formation during Cu^{2+}/H_2O_2 treatment of RNase and lysozyme suggests that Tyr residues may be adjacent to these metal binding sites.

Visible wavelength, alkaline blue fluorescence also increased steadily in RNase and lysozyme during exposure to oxidative stress, and this commonly termed DT-like fluorescence did have excitation and emission maxima similar to those of DT. Earlier reports in the literature (25–27) have implied that DT is a significant contributor to the non-tryptophan fluorescence that develops during protein oxidation, and we observed that DT was the major non-tryptophan fluorophore produced during MCO of proteins, accounting for 100% and 50% of the total protein fluorescence in RNase and lysozyme (Fig. 5*B*), respectively. In contrast, DT was a minor contributor to protein fluorescence formed during radiolytic oxidation of RNase and lysozyme, accounting for only 16% and 2% of the fluorescence in the proteins at a dose of 75 kilorads. The contribution of DT to total fluorescence was greater for RNase, which lacks Trp, than for lysozyme which contains 6 mol of Trp/mol of protein (Fig. 5).

The above results have shown that both *o*-Tyr and DT are markers of metal-catalyzed oxidative damage to protein. In addition, DT has been shown to account for a large fraction of the fluorescence that accumulates in protein upon exposure to Cu^{2+}/H_2O_2. These findings, coupled with the physiological significance of MCO reactions, indicated that *o*-Tyr and DT should be useful as biomarkers of oxidative damage to tissue proteins and that DT may account for a sizeable fraction of the non-tryptophan fluorescence that accumulates in long-lived proteins exposed to MCO *in vivo*. In the accompanying

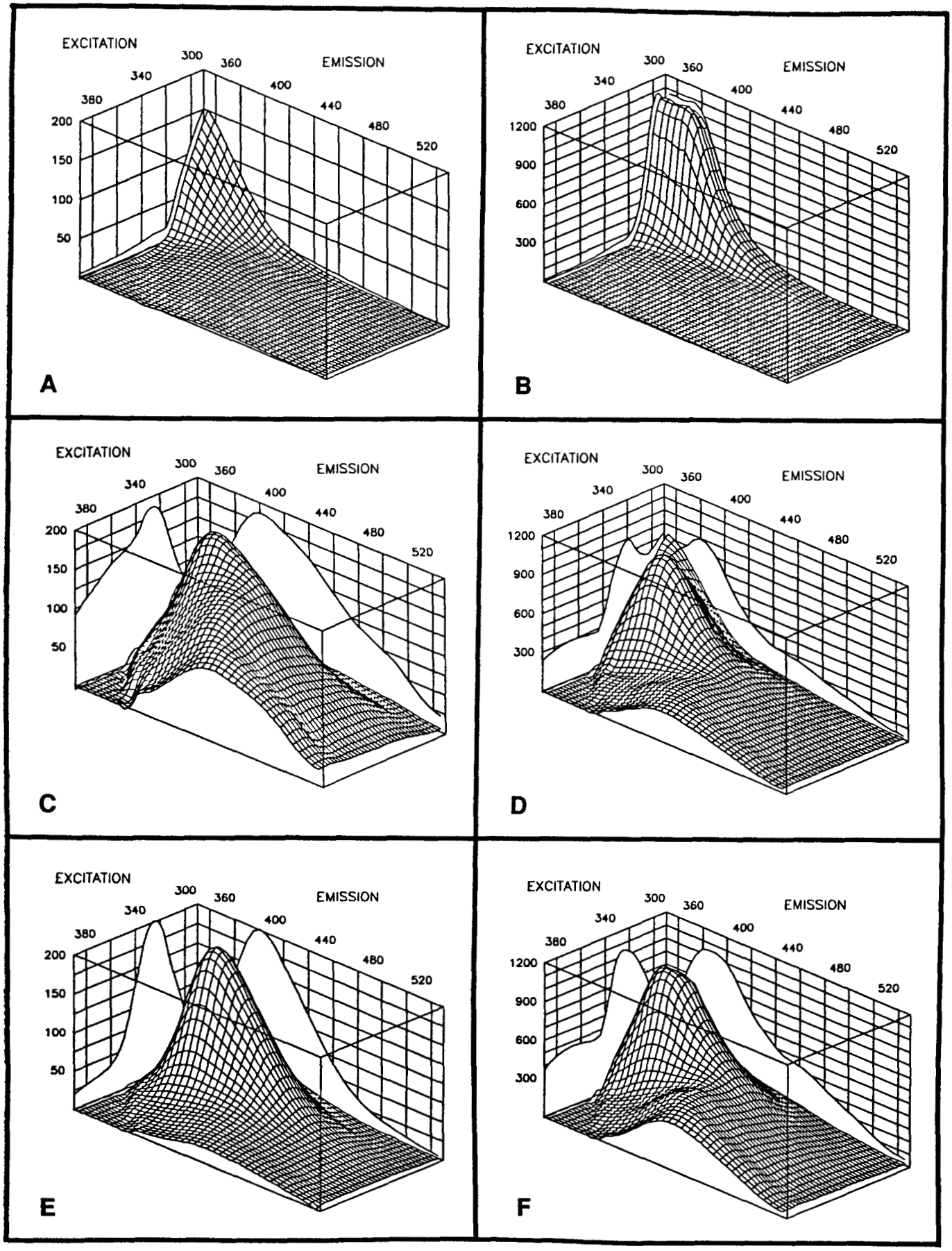


FIG. 6. **Three-dimensional fluorescence spectra of control and oxidized RNase and lysozyme.** Protein samples (1 mg/ml) were solubilized with chymotrypsin, and three-dimensinal fluorescence spectra were recorded as described under "Experimental Procedures." *A* and *B*, native RNase and lysozyme, respectively; *C* and *D*, RNase and lysozyme, respectively, irradiated with a dose of 45 kilorads; *E* and *F*, RNase and lysozyme, respectively, oxidized with 2.5 mM H_2O_2.

paper (14), we used these biomarkers to assess the role of MCO reactions in the aging of lens proteins.

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